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Enzyme-Linked Immunosorbent Assay for Detection of *Salmonella typhi* Vi Antigen in Urine from Typhoid Patients

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Because typhoid fever continues to be a major cause of illness in many developing countries, there is a clear need for a sensitive and specific test that will permit rapid laboratory diagnosis of the disease. An enzyme-linked immunosorbent assay (ELISA) has recently been developed and tested, both in the laboratory and in a clinical situation, for its ability to detect Vi antigen in urine. The ELISA was capable of detecting as little as 1 ng of purified Vi antigen per ml in urine, compared with 100 ng/ml detectable by a previously tested coagglutination method. It could also detect antigen in urine diluted as much as 1:1,024 in normal urine. In tests of urine specimens from six stool culture-positive persons in a small typhoid outbreak in the United States, the ELISA detected antigen in specimens from four of the six patients. The ELISA also proved to be specific, giving no false-positive results for specimens from 50 persons who did not have typhoid fever. The apparent high sensitivity and specificity of this ELISA make it a promising test for rapid diagnosis of typhoid fever.

Despite its diminishing incidence in the United States and Europe, typhoid fever remains a major cause of illness in many parts of the developing world. The diagnosis of typhoid fever in these areas is frequently made solely on clinical grounds. Blood and stool cultures are done in relatively few hospitals, and many cultures are falsely negative due to prior antibiotic treatment. Even when appropriate cultures are taken, presumptive bacteriological results require 1 or 2 days, and confirmation requires even longer.

Serological diagnosis of typhoid fever (the Widal test) has been found by some investigators to be unreliable (3, 8). In areas with endemic typhoid, serodiagnosis requires both acute and convalescent sera, since the prevalence of O and H antibodies in the adult and adolescent populations is high (3). In this case, diagnosis is made purely retrospectively.

Clearly, there is a need for a sensitive and specific test that will permit rapid laboratory diagnosis of typhoid fever. Rockhill et al. have reported a test that detected soluble *Salmonella typhi* antigens Vi, D, and d in the urine of Indonesian patients with typhoid fever (5). Antibodies to these antigens were attached to protein A-rich staphylococci, and antigen was detected by mixing urine with the antibody-coated cells on a slide and observing for coagglutination. The test showed high sensitivity (59 of 61 urine samples from culture-proven typhoid patients were positive) but low specificity (8 of 46 control urines were positive).

This paper describes an enzyme-linked immunosorbent assay (ELISA) for the detection of Vi antigen in urine and discusses its sensitivity and specificity in laboratory experiments and its application in investigating a small typhoid outbreak in the United States.

MATERIALS AND METHODS

Reagents. Burro anti-*S. typhi* TY-2 plasma was the generous gift of John B. Robbins, Bureau of Biologics, Food and Drug Administration. Highly purified Vi antigen, prepared from *Citrobacter* 5396/38 as previously described (9), was provided by Kwei-Hay Wong, Bacteriology Division, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Ga. Rabbit anti-Vi antiserum was obtained from the Biological Products Division, Centers for Disease Control, and the immunoglobulin G (IgG) fraction was separated by DEAE-Sephadex chromatography. Goat anti-rabbit IgG was purchased from Antibodies, Inc. (Davis, California) and coupled to alkaline phosphatase (Sigma Chemical Co., St. Louis, Mo.) by the method of Voller et al. (6).

ELISA procedure for detection of Vi antigen. Optimal dilutions of all reagents were determined by checkerboard titration, and optimal times and temperatures for incubation were determined before assays were carried out. Burro anti-*S. typhi* TY-2 was diluted 1:5,000 in 0.06 M carbonate buffer (pH 9.6), and 0.1 ml was added to alternate rows of the inner wells of a polyvinyl U-bottomed microtiter plate (Dynatech Laboratories, Inc., Alexandria, Va.). Unimmunized burro

serum, diluted 1:5,000 in carbonate buffer, was added to the other rows of wells. The plates were incubated overnight in a moist chamber at 4°C. Plates were then washed three times with phosphate-buffered saline (PBS), pH 7.2, with 0.05% Tween 20, and 0.05 ml of PBS-Tween 20 with 1% fetal calf serum was added to each well.

To determine the sensitivity of the test, 10-fold dilutions of purified Vi antigen (1 mg/ml to 1 pg/ml) were made in Vi ELISA-negative urine, and 0.05 ml of each dilution was added to duplicate wells of both normal serum-coated and immune serum-coated rows. For testing samples, 0.05 ml of each urine specimen was added to duplicate wells of each type.

Plates containing samples were incubated for 4 h at 37°C and were then washed three times in PBS-Tween 20. Rabbit anti-Vi IgG was diluted 1:1,000 in PBS-Tween 20, and 0.1 ml was added to each well. After 1 h of incubation at 37°C, plates were again washed three times in PBS-Tween 20. Alkaline phosphatase-conjugated goat anti-rabbit IgG was diluted 1:200 in PBS-Tween 20 and added in 0.1-ml amounts to each well. Plates were incubated for 1 h at 37°C and washed three times with PBS-Tween 20. *p*-Nitrophenyl phosphate (Sigma) in diethanolamine buffer, 1 mg/ml, was added, the plates were incubated at room temperature for 30 min, and the reaction was terminated by adding 0.025 ml of 3 N NaOH to each well.

Results were read visually. Development of a yellow color in wells coated with immune serum and not in the corresponding wells coated with normal serum was considered a positive test for Vi antigen.

Coagglutination. Rabbit anti-Vi IgG (the same lot prepared and used as described above) was coupled to protein A-rich staphylococci, and slide coagglutination tests were performed on the same materials tested by ELISA. Materials and methods for the test were the same as those described by Rockhill et al. (5), except our preparation of rabbit anti-Vi IgG was used instead of Difco antiserum.

O, H, and Vi antibodies. Patient serum specimens were tested for O and H (2) and Vi (4) antibodies by previously described methods.

Clinical specimens. The investigation of a typhoid outbreak associated with attending a weekend of social functions in a rural Missouri county in May, 1980, provided an opportunity to use the ELISA for Vi antigen to test clinical specimens. Urine and serum specimens were obtained 39 to 41 days postexposure from 6 culture-positive and 10 well persons who had attended the social functions. Urine samples were tested for Vi antigen, and serum sample titers were determined for Vi, O, and H antibodies. Additional urine samples were obtained from 21 persons with urinary tract infections seen at an Atlanta clinic, 19 presumably healthy persons at the Centers for Disease Control, and one person with acute typhoid fever not associated with this outbreak. All serum and urine specimens were frozen promptly upon collection and held at −20°C until being tested 5 to 7 days later.

RESULTS

Sensitivity. With the use of purified Vi antigen diluted in normal urine, the ELISA consistently detected 1 ng of antigen per ml. The coagglutination method detected antigen down to a concentration of 100 ng/ml. When urine from a patient with typhoid fever who was not part of this outbreak was diluted in normal urine, the ELISA detected Vi antigen in urine diluted 1:1,024, whereas the coagglutination method was positive only to the 1:32 dilution.

Specificity. None of the urine samples from the 10 well persons who attended the social functions and none of the 40 urine specimens from persons not involved in this outbreak gave a positive reaction in the ELISA. However, 2 of these 50 samples produced color in both normal serum- and immune serum-coated wells, making the test uninterpretable. Positive coagglutination reactions were observed in 0 of 10 samples from exposed persons and 6 of 40 samples from unexposed persons.

Clinical data. Vi antigen was detected in the urine of four of six culture-positive persons by the ELISA and in one of the six persons by coagglutination (Table 1). None of 10 controls was positive by either test. Four of six culture-positive persons had elevated (≥20) Vi antibody titers, four of six had elevated (≥160) H agglutination titers, and four of six had elevated (≥160) O agglutination titers. None of the controls had elevated Vi, O, or H antibody titers. Five of the six infected persons had completed a course of antibiotic therapy before urine and serum specimens were obtained.

TABLE 1. Laboratory results of urine and serum specimens from *S. typhi*-infected persons

Person	Days after onset specimens obtained	Reciprocal titers of following antibody:			Vi antigen in urine by following test:	
		Vi	O	H	ELISA	Coagglutination
1	35	<10	160	<20	−	−
2	29	640	≥640	160	+	−
3	32	10	80	640	+	−
4	33	320	320	320	+	−
5	32	1,280	≥640	≥640	+	+
6[a]		40	20	20	−	−

[a] Person 6 was asymptomatic and a possible chronic carrier.

One of the two infected persons who did not have Vi antigen detected in urine had the longest interval between onset of symptoms and the collection of urine (35 days). The other Vi antigen-negative person had not been ill and had low O and H antibody titers, indicating a probable chronic carrier state in which circulating antigens would not usually be expected. The only person whose urine was positive by both tests for Vi antigen was the only person who was known to have a culture-positive stool at the time the urine samples were collected.

DISCUSSION

The sensitivity of the ELISA in detecting Vi antigen (1 ng/ml) is comparable to the reported sensitivites of ELISAs for other bacterial antigens in body fluids (7). The sensitivity we observed for the coagglutination test (100 ng/ml) is also similar to the sensitivity of the coagglutination test for *Escherichia coli* heat-labile enterotoxin reported by Brill et al. (1). The 100-fold greater sensitivity of the ELISA proved valuable in testing clinical specimens, since four of six urine samples were positive by the ELISA, whereas only one of six was positive by coagglutination.

Since 0 of 10 urine specimens from non-ill exposed persons and 0 of 40 urine specimens from persons who were not exposed to *S. typhi* gave positive results, the specificity of the test appears to be excellent. Two (4%) of these urine specimens had nonspecific reactions that made the tests impossible to interpret. The reason for these nonspecific reactions was not identified.

Our clinical samples were unusual, since all were collected at least 1 month after onset of illness (Table 1). The lateness of collection almost certainly reduced the chance of finding Vi antigen in the urine to below the chance of finding it in a hospital setting, where specimens would normally be obtained from acutely ill persons 5 to 7 days after onset. Since the coagglutination test is less sensitive than the ELISA, delay in specimen collection may have affected the coagglutination test results more than the ELISA results. Only one person was known to be still excreting *S. typhi* at the time of urine and serum collection, and his urine was positive by the coagglutination test as well as by the ELISA. This person also had the highest level of Vi antibodies in serum of the six tested.

The number of persons who would have been diagnosed as having had typhoid fever on the basis of Vi antigen in urine (four of six) was similar to the number who would have been diagnosed by elevated levels of Vi (four of six), O (four of six), or H (four of six) antibodies. The similarity of these test results may not be observed in areas with endemic typhoid, where the prevalence of O and H antibodies is high in the normal population (3).

The apparent high sensitivity and specificity of the ELISA for Vi antigen in urine make it a promising test for the rapid diagnosis of typhoid fever. An evaluation of its usefulness in an area with endemic typhoid is being planned. Simultaneous evaluation of the coagglutination test is also planned, since the greater sensitivity and specificity of the more cumbersome ELISA may not prove to be as advantageous in a hospital setting as our current data imply.

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